

*SEPARATION OF THE RNA MESSAGE TRANSCRIBED IN
RESPONSE TO A SPECIFIC INDUCER**

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The addition of an inducer such as thio-methyl- β -D-galactoside (TMG) to a wild-type strain of *Escherichia coli* specifically stimulates the synthesis of the enzyme β -D-galactosidase.^{1, 2} An attempt at elucidating the mechanism of this process is the object of the present paper.

Two sites of inducer action may be proposed in terms of the currently accepted view of information flow from the genome to the protein-synthesizing machines. One site would be at the gene level, the inducer releasing an inhibition of the transcription mechanism that produces complementary RNA copies of the gene. The other site would be at the level of translation of the genetic messages into protein molecules. Although these alternatives are not mutually exclusive, their individual operation can be tested since techniques are available to provide an answer to the following question: *Does the presence of inducer increase the production of message homologous to the relevant genetic region?*

The detection and assay of RNA complementary to the genetic region responsible for β -galactoside utilization—the *lac* region—can, in principle, be carried out by the hybridization test of Hall and Spiegelman.³ A purified preparation of the corresponding DNA segment would furnish optimal hybridizing material for this RNA. An approximation is provided by the bacteriophage P1*dl*, a high-frequency transducing phage which has been shown to carry the *lac* “operon.”^{4, 5} The use of the DNA from this phage as a detecting device introduces some uncertainties due to possible homologies involving segments of the genome other than the *lac* region. Fortunately, the uncertainties introduced by extraneous homologies can be minimized by another device. Kano-Sueoka and Spiegelman⁶ showed that, given two RNA preparations, components present in only one of them can be identified by using two different isotopic labels and simultaneous chromatography. If induction stimulates message production, RNA unique to induced cells should be detectable by chromatographic fractionation, which may also separate it from other RNA fractions that happen to be complementary to the detector DNA.

The present paper describes experiments which demonstrate the presence in induced cells of increased amounts of RNA complementary to the DNA of the *lac*

region. This *lac*-RNA can be identified chromatographically and distinguished from other *E. coli* RNA messages which possess homology for the bacteriophage genome. The data are consistent with the concept that the presence of inducer leads to an increased rate of message production from the *lac* region of the genome.

In two recent preliminary accounts, Attardi *et al.*⁷ reported experiments in which the fraction of RNA message corresponding to the galactose (*gal*) region was detected by hybridization between unfractionated *E. coli* RNA and the DNA from the transducing phage λ *dg*. Their findings are amplified, and their conclusions on the site of induced action confirmed, by the present results.

Materials and Methods.—*Bacterial and viral strains:* Two strains of *E. coli* were used as sources of RNA. One was the inducible strain BB, $i^+z^+y^+$, wild-type; the other was strain W4032 $i^-z^-y^-$, which is a *lac*⁻ deletion mutant⁸ derived from *E. coli* K-12.

The phage strains used were P1 and one of the defective P1*dl* phages that transduce the *lac* genes at high frequency.⁴ The particular P1*dl* employed was derived from P1*dl*₆₀,⁵ which carries the *lac* region of *Shigella dysenteriae*, strain 60, $i^+z^+y^{del}$. Recombination occurring in a strain of *E. coli* carrying P1*dl*₆₀ as well as a *coli lac* region $i^-z^-y^+$ gave rise to a line of P1*dl*, $i^-z^+y^{del}$, in which the genes i^- and z^+ from *E. coli* have replaced most of their homologues from strain 60 (N. C. Franklin and B. Howard, unpublished). This P1*dl* line was chosen for the present work because it consistently yields high titers of transducing phage particles.

E. coli strain W4032 was used to grow P1 and *S. dysenteriae* strain Sh to assay it. For preparation of P1*dl* lysates, cultures of W4032 (P1*dl*) were induced with ultraviolet light, superinfected with P1, and allowed to lyse. The lysates, with titers of about 2×10^8 P1 and 10^8 transductions per ml, were filtered; the phage was concentrated 400-fold by 2 cycles of high and low speed centrifugation and was resuspended in phosphate buffer 0.1 M, pH 7.0, with 0.1 M NaCl and 0.01 M MgSO₄ added. Each phage preparation was assayed for plaque-forming units and for transduction units. The transduction titers are underestimations of the titers of P1*dl* in these preparations; additional estimates were obtained from the rates of β -galactosidase synthesis observed after infecting *E. coli* z^-y^+ bacteria with known aliquots of each P1*dl* preparation.⁹ Since phages P1 and P1*dl* cannot be separated by density-gradient centrifugation^{5, 10} their relative concentrations in each preparation had to be calculated from these rather crude estimates. Hence, the corrections used in Tables 1 and 2 must be considered as only rough approximations.

TABLE 1
HYBRIDIZED BULK RNA AS RNAASE-RESISTANT COUNTS IN THE DNA DENSITY REGION

Source of RNA	DNA Preparation		
	P1 <i>dl</i>	P1	P1 <i>dl</i> corrected for P1
W4032 "induced"	31	58	0
BB induced	280	106	216
BB noninduced	72	87	25

Induction, pulsing with H³-uridine, purification of RNA, and assay of RNAase-resistant cpm were as described under *Methods*. The first column represents the sum of counts hybridized to P1*dl* DNA, as shown in Figure 1; the second column is from a similar experiment with P1 DNA. In all cases the number of counts included in the hybridizing mixture is the same. The third column is obtained as follows: Column 1—column 2 (0.6×0.9). The 0.6 factor is an estimate of the fractional content of P1 in the preparation of P1*dl*; the 0.9 factor adjusts for a difference in the DNA content of the two preparations as determined from O.D.₂₆₀.

TABLE 2
HYBRIDIZATION OF PREFRACTIONATED RNA TO DNA

Source of Region R ₂ RNA	DNA Preparation		
	P1 <i>dl</i>	P1	P1 <i>dl</i> corrected for P1
BB induced	594	151	514
BB noninduced	84	125	16

RNA from a noninduced BB culture was fractionated and a sample corresponding to region R₂ of Figure 3 was collected and concentrated. Its ability to hybridize with P1*dl* DNA and P1 DNA was compared under identical conditions to that of a similar sample of RNA from an induced BB culture. Conditions and calculations are as in Table 1.

Phage DNA was purified by the method of Grossman *et al.*¹¹ Heat denaturation of the DNA was carried out as described previously.¹²

RNA preparation: Bacteria were grown in SC medium,¹³ with glycerol as a carbon source, plus 0.05% of casein hydrolysate and 10 γ /ml of thiamine. Bacteria were pulsed with radioactive uridine for 90 sec during log phase. The RNA was extracted by the phenol method and purified as detailed by Hayashi and Spiegelman.¹³ The purified RNA was concentrated by the procedure of Yankofsky and Spiegelman.¹²

Column chromatography: The preparation of the methylated albumin columns and their use for RNA fractionation follow the detailed description of Kano-Sueoka and Spiegelman.⁶

DNA-RNA hybridization and assay of hybridized material: The buffer and ionic conditions used for hybridization are as reported previously.¹² The reaction mixtures containing in 0.6 milliliters 20 γ of heat-denatured DNA and fixed amounts of RNA were incubated at 43°C for 24 hr. The samples were then placed in CsCl and centrifuged to density equilibrium in the SW 39 swinging bucket rotor of a Spinco Model L centrifuge.³ Samples collected dropwise from the bottom of the tube were diluted to 1 ml with buffer and the O.D. at 260 $m\mu$ was determined. Aliquots were diluted to 10 ml with Tris-Mg⁺⁺-NaCl buffer ($3 \times 10^{-2} M$, $5 \times 10^{-2} M$, 0.03 M , respectively), pH 7.4. The diluted samples were treated with pancreatic RNAase (10 γ /ml) at 30°C for 20 min. As an internal control of the digestion, P³²-labeled 23S ribosomal RNA was added (a correction of the order of 3% being made for the RNAase-resistant core of the added RNA). Procedures for counting the RNAase-resistant radioactive material on millipore membranes in a liquid scintillation spectrophotometer have been described.¹²

To check the adequacy of the hybridization conditions as an assay system, a series of preliminary tests was carried out with heat-denatured P1*dl* DNA and labeled RNA from induced BB. The DNA was kept constant at 20 γ per 0.6 ml sample, the input RNA was varied, and the amount of cpm hybridized was determined. The amount of RNA hybridized was linearly related to the RNA input up to levels twice as high as those employed in the present study. This ensures that the DNA was present in adequate excess.

Reagents: H³-uridine at 500 $\mu\text{c}/112 \gamma$ from New England Nuclear Corp. and C¹⁴-uridine at 5.7 $\mu\text{c}/\text{mgm}$ from Schwarz BioResearch, Inc. were used. For usual labeling the H³-uridine was diluted 10-fold. When RNA of high specific activity was required, the H³-uridine was used undiluted. In all cases 1 γ uridine per ml was present. The inducers TMG and isopropyl-thio- β -D-galactoside (IPTG), obtained from Mann Research Laboratories, were used at $5 \times 10^{-3} M$.

Results.—As already indicated, the question of inducer effect can be examined in two ways. In the first, the amounts of RNA specifically complementary to the DNA from P1*dl* is compared by hybridization with bulk RNA from induced and noninduced cultures. In the second, an RNA fraction specific to induced *lac*⁺ cells is located by a direct chromatographic separation and its message nature is checked by hybridization tests.

A. *Hybridizations with unfractionated RNA:* The insertion of suitable controls required the preparation of labeled RNA from: (a) an induced wild type; (b) a noninduced wild type; (c) an “induced” *lac* deletion. Accordingly, log-phase cultures of *E. coli* BB and W4032 were pulsed with H³-uridine for 90 sec after five min of growth with inducer; a similar pulse was performed on *E. coli* BB without inducer. The radioactivity in the bulk RNA from these cultures was 8.6×10^5 cpm/mg, 8.3×10^5 cpm/mg, and 8.4×10^5 cpm/mg for the induced BB, induced W4032, and noninduced BB, respectively.

Results of hybridization between these preparations and P1*dl* DNA are described in Figure 1. It is evident that little hybrid is formed with the RNA from the *lac*-deletion mutant and that the largest amount of hybrid is observed when the RNA is derived from the induced wild-type culture. For quantitative interpretation, one must take into account that phage preparations of P1*dl* always contain some phage P1. The best available estimate for the P1*dl* preparation used in the present

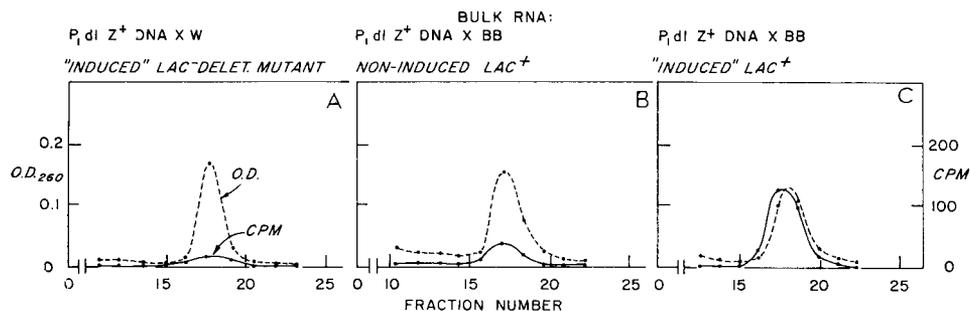


FIG. 1.—RNAase-resistant counts in DNA density region. (A) Bulk RNA from strain W4032 (*lac* deletion) labeled with H³-uridine between 5 and 6.5 min after induction with IPTG. (B) Bulk RNA from BB labeled during the same period without inducer. (C) Bulk RNA from BB labeled during the same period with inducer. Mixtures of heat-denatured P1 *dl* DNA and bulk RNA were made in TMS buffer (0.03 M Tris, pH 7.3, 0.001 M MgCl₂, 0.3 M NaCl) and kept at 43°C for 24 hr; they were then brought to a density of 1.73 and a total volume of 5 ml with CsCl. Centrifugation was for 70 hr at 33,000 rpm at 25°C. Fraction collection and counting of RNAase-resistant material as described in *Methods*. The amounts of H³-RNA and of DNA included in reaction mixture were the same in all cases.

study indicates that P1 represented about 60 per cent of the total phage present. Hence, parallel control hybridizations were carried out with P1 DNA. The results not only served as a check on the nature of the DNA-RNA hybrids formed with P1 *dl* DNA, but also permitted corrections for extraneous hybrid formation with P1 DNA. Table 1 summarizes the results of hybridizations with both P1 and P1 *dl* DNA. Comparing the first two columns we note that RNA from induced BB contains approximately four times more RNA complementary to the P1 *dl* DNA preparation than the noninduced control. When tested with DNA from P1, the two preparations show only a slight difference. The RNA from the "induced" deletion mutant W4032 contains little RNA hybridizable to the P1 *dl* DNA.

Since some hybridization between *coli* RNA and P1 DNA occurs, a correction must be applied in order to estimate the amount of RNA specifically hybridizable to P1 *dl*. This is done in the third column of Table 1. From the corrected values it appears that virtually all of the material which hybridizes to P1 *dl* from RNA of the "induced" deletion mutant is accounted for on the basis of P1 contamination. In addition, the correction increases the difference in hybridization between the RNAs of induced and noninduced BB to a factor of about 10. Attardi *et al.*⁷ also found that *E. coli* RNA gives some hybridization with the DNA from phage λ .

The data of Table 1 as well as the results with the *gal* region⁷ support a mechanism of inducer function which results in an increased production of RNA complementary to the genetic region whose product the inducer increases. This conclusion was further tested by a different procedure, which in addition permits resolution of ambiguities introduced by the background of irrelevant homologies.

B. Chromatographic identification of the lac message: The idea underlying the method can be stated simply. Consider two labeled RNA preparations, one identified by H³ and the other by C¹⁴. If a mixture is loaded on a column, the elution profiles of the H³ and C¹⁴ labels should be identical if the two preparations are the same, and should differ if one contains some components absent from the other.

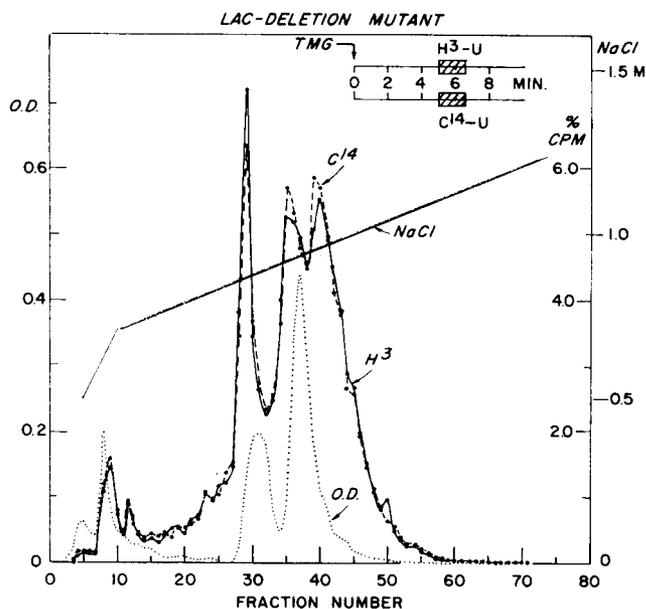
The problems at hand can be answered using mixtures of RNA purified from the following three pairs of cultures: (A) two wild-type, inducible BB cultures, one

labeled with H^3 -uridine in the presence of inducer, the other with C^{14} -uridine in its absence; (B) two BB cultures, both without inducer, one labeled with H^3 , the other with C^{14} -uridine; (C) two W4032 cultures (*lac*-deletion), one labeled with H^3 -uridine in the presence of inducer, the other with C^{14} -uridine in its absence.

In mixture (A), an inducer-stimulated production of *lac* message will be signaled by the appearance in the H^3 profile of components which are absent in the C^{14} profile. These discordancies should be absent from elution profiles of mixtures (B) or (C). In mixture (B) neither culture contained inducer during labeling; this mixture also serves to monitor the reproducibility of the profiles. Mixture (C) serves to detect irrelevant effects of inducer on RNA synthesis since in this instance the *lac* region is missing.

We first examine the two control preparations. Figure 2 describes the profiles of the RNA from the *lac*-deletion mutant. The presence of inducer causes no significant distortion in the distribution of one isotope with respect to the other. The excellent concordance of C^{14} and H^3 in the other control (uninduced BB, Fig. 3A) proves that the various steps involved are reproducible in terms of the final outcome. (A comparison of the profiles of Figures 2 and 3 would not be justified, because strains BB and W4032 are independently isolated wild-type strains.)

FIG. 2.—Column chromatography of RNA from cultures of *lac*-deletion mutant with and without inducer. As in all the other figures, protocols and timings are diagrammed. One culture was induced with IPTG at 0 time and pulsed with H^3 -uridine at 5 min. The other culture, without inducer, was pulsed with C^{14} -uridine. At 6.5 min, incorporation was terminated; RNA was isolated from each culture and purified. A mixture of the two samples was chromatographed. O.D. profile identifies pre-existent stable components. NaCl gradient, measured by refractive index, is indicated. Here and in Fig. 3, counts are expressed as per cent of total to permit easier visual comparison of profiles.



The experimental mixture is shown in Figure 3B. Here we note two discordancies of the induced RNA (H^3 -profile, region R_2), which are reproducibly observed when induced and noninduced RNA from an inducible strain are compared.

Direct evidence that these discordancies correspond to messages from the *lac* region is provided by hybridization between P1*dl* DNA and various RNA fractions. To reduce the experiment to manageable proportions, fractions within the regions indicated in Figure 3A as R_0 , R_1 , R_2 , and R_3 were pooled, concentrated, and used in the hybridization test. The RNAase-resistant counts found in the DNA density region of a CsCl gradient are shown in Figure 4. It is clear that a major portion

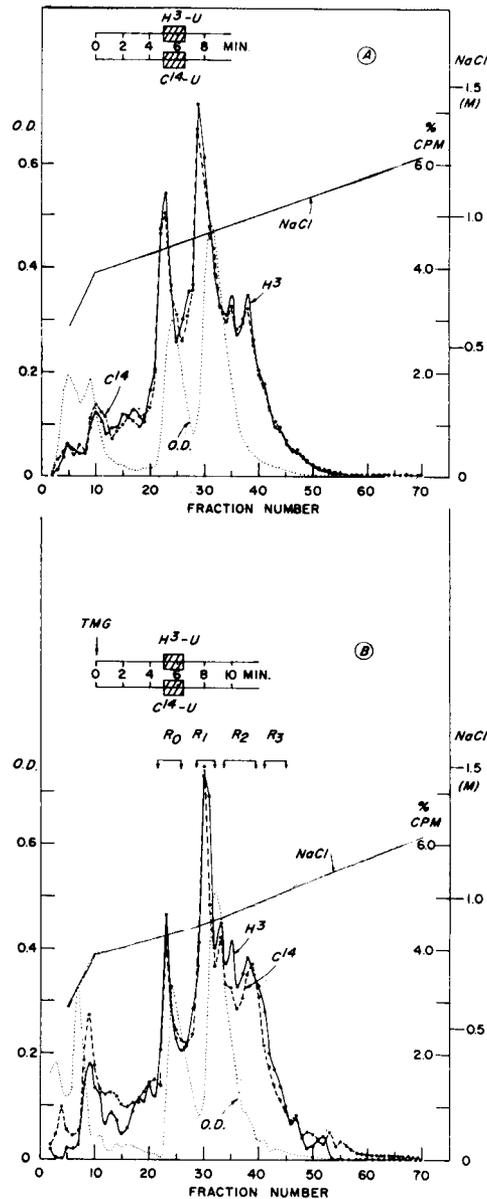


FIG. 3.—Column chromatography of induced and noninduced RNA from wild type. (A) Control mixture of two RNA samples of *E. coli* BB independently labeled during the same period, without inducer. (B) Mixture of induced and noninduced RNA samples. H³ labels the induced culture, C¹⁴ the noninduced culture. All symbols and other details as in Fig. 2.

inducer had virtually no effect on the amount of RNA complementary to DNA from a P1 phage which does not contain the *lac* genes. The second control showed that

of the RNA hybridizable to P1*dl* DNA is found in region R₂, which corresponds to the discrepant bands observed in the H³ profile of Figure 3B.

The column fractionation permits differentiation between the RNA complementary to P1 DNA and the RNA that specifically hybridizes to P1*dl* DNA. Figure 5 summarizes the extent of hybridization with P1*dl* and P1 DNA in terms of the cpm complexed in the different regions of the column effluent. The RNA components which hybridize with P1*dl* DNA appear to have a peak at a different position from those components that can hybridize with P1 DNA. Unfortunately, beyond region R₃ the counts were too few for use.

Since most of the RNA specific for the *lac* region is to be found in region R₂, a series of hybridizations was carried out with RNA from this region derived from both induced and noninduced cultures. Again, the same number of counts and amounts of DNA were included in the reaction mixtures. The results are summarized in Table 2. Comparison with the bulk hybridizations of Table 1 reveals that the use of the fractionated RNA has magnified the difference between the induced and noninduced preparations. Before adjustment for P1 content the difference is 7-fold; after correction it is over 30-fold.

Discussion.—Hybridizations with DNA from the transducing phage P1*dl* indicate that an inducer of galactosidase synthesis increases the production of RNA complementary to the *lac* region DNA. Controls of two types were used to monitor the meaningfulness of the observed stimulation. The first control showed that

addition of inducer to a *lac*-deletion mutant of *E. coli* did not result in the appearance of an RNA which hybridizes specifically with P1*dl* DNA.

An independent confirmation of these observations was made possible by simultaneous column chromatography, which revealed in RNA from induced cells the presence of components not detectable in noninduced controls. Hybridization of various fractions with P1*dl* DNA confirmed that the distinctive RNA peaks found in the induced preparation contained RNA complementary to the *lac* region. Parallel tests with P1 DNA revealed the existence of *E. coli* RNA messages external to the *lac* region and which can hybridize with P1 DNA. These could be distinguished on the column from those that hybridize with P1*dl* DNA.

Estimates from hybridizations with fractionated RNA suggest that the presence of an inducer increases 30-fold the amount of RNA complementary to the *lac* region. It must be emphasized that this is only a rough approximation which may underestimate the true value by an order of magnitude.

The conclusions that can be drawn with respect to inducer function may briefly be mentioned. It will be noted that we have not considered the possibility that inducer functions by preventing destruction of message. Such mechanisms are improbable in view of the cogent genetic arguments developed by Jacob and Monod.¹⁴ Further it must be recalled that the increases in *lac* RNA were observed in cultures that were actively synthesizing enzyme and presumably, therefore, using

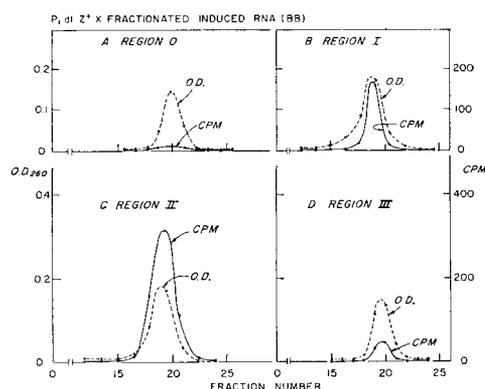
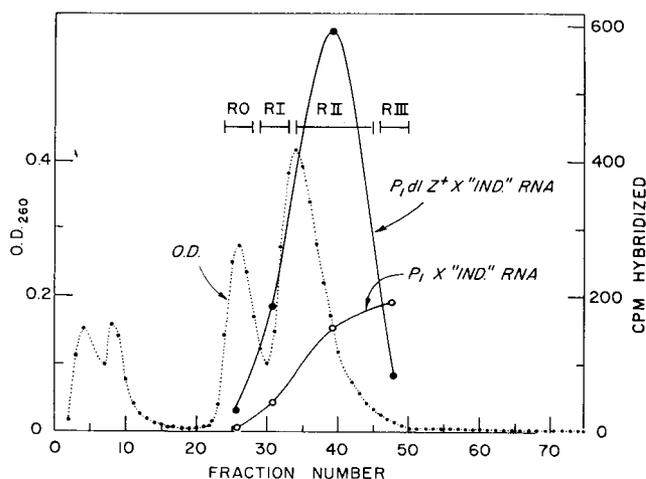


FIG. 4.—Hybridization of fractionated induced RNA. A culture was induced and labeled with H^3 -uridine at high specific activity ($500 \mu\text{C}/112 \gamma$), and fractionated as in the experiment of Fig. 3B. Fractions corresponding to the 4 regions indicated were pooled, concentrated, and aliquots were used for hybridization. All other details as in Fig. 1. Here and in Fig. 5 the regions 0, I, II, III correspond to regions R_0 , R_1 , R_2 , R_3 in Fig. 3 and text.

Fig. 5.—Column separation of *lac* RNA and of RNA complementary to P1. For each region the amounts of RNA found to be hybridizable to P1*dl* DNA and to P1 DNA are plotted. The O.D. profile is reproduced to provide a ready identification of relative positions. The data from Fig. 4 were used for the P1*dl* DNA-curve; those for the P1 DNA curve are from a parallel experiment.



the corresponding message. Any mechanism which would restrict inducer function to the site of message translation into protein is made unlikely by the present data. These findings do not eliminate, however, a double effect on both production and use of message. Neither do they specify that the *primary* site of inducer function is at the transcription step since this step may be stimulated indirectly. Thus, the inducer may inactivate a repressor or inhibit its production. The experiments do indicate that, whatever the detailed mechanism, the presence of inducer does result in an increased transcription of the relevant genetic region.

The present experiments were done with a single type P1*dl*. Others can be prepared, carrying either complete or partially deleted *lac* regions, which can be used for further characterization of the specifically complexed RNA messages.

Summary.—Hybridization of RNA with DNA from a transducing phage carrying the *lac* genetic region and chromatographic fractionation of RNA were employed to detect the specific *lac* message RNA in *E. coli*. The experiments demonstrate the occurrence in induced cells of increased amounts of RNA messages complementary to the *lac* region. This RNA can be identified chromatographically and distinguished from other *E. coli* messages which possess homology for some portions of the phage genome. The data are consistent with the conclusion that the presence of inducer leads to an increased rate of transcription from the *lac* region.

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